Microfluidic Chip for Detection of Fungal Infections

Waseem Asghar,*,[†,‡] Mazhar Sher,[†,‡] Nida S. Khan,[§] Jatin M. Vyas,[§] and Utkan Demirci*,[∥]

[†]Ashgar Lab, Micro and Nanotechnology in Medicine, College of Engineering and Computer Science, Boca Raton, Florida 33431, United States

[‡]Department of Computer & Electrical Engineering and Computer Science, Florida Atlantic University, Boca Raton, Florida 33431, United States

[§]Division of Infectious Disease, Harvard Medical School, Massachusetts General Hospital, Boston, Massachusetts 02115, United States

[∥]Bio-Acoustic MEMS in Medicine (BAMM) Laboratory, Canary Center at Stanford for Cancer Early Detection, Department of Radiology, School of Medicine, Stanford University, Palo Alto, California 94305, United States

Ⓢ Supporting Information

ABSTRACT: Fungal infections can lead to severe clinical outcomes such as multiple organ failure and septic shock. Rapid detection of fungal infections allows clinicians to treat patients in a timely manner and improves clinical outcomes. Conventional detection methods include blood culture followed by plate culture and polymerase chain reaction. These methods are time-consuming and require expensive equipment, hence, they are not suitable for point-of-care and clinical settings. There is an unmet need to develop a rapid and inexpensive detection method for fungal infections such as candidemia. We developed an innovative immuno-based microfluidic device that can rapidly detect and capture *Candida albicans* from phosphate-buffered saline (PBS) and human whole blood. Our microchip technology showed an efficient capture of *C. albicans* in PBS with an efficiency of 61−78% at various concentrations ranging from 10 to 10^5 colony-forming units per milliliter (cfu/mL). The presented microfluidic technology will be useful to screen for various pathogens at the point-of-care and clinical settings.
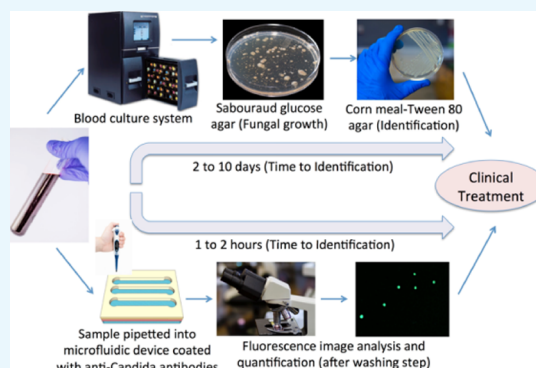
1. INTRODUCTION

Candida albicans (*C. albicans*) are human commensals of genitourinary and gastrointestinal tracts, and skin.[1] However, *C. albicans* is an opportunistic fungal pathogen that can cause invasive fungal infections.[2,3] Mortality associated with *C. albicans* infection is greater than 50% making *Candida* a leading cause of healthcare-associated bloodstream infections in the United States.[3] Neutrophils are an essential element of the innate immune system. Low count of neutrophils in blood (neutropenia) and its lengthy and repeated episodes provide favorable conditions for *Candida* invasion, especially in immunocompromised patients.[4] Because of the lack of rapid diagnosis, these bloodstream infections require prolonged hospital stays that significantly increase treatment and hospitalization costs.[3,5,6]

Rapid detection of *Candida* is urgently needed, especially in the cases of preterm neonates and immunocompromised patients; candidemia can lead to multiple organ failure and septic shock.[7−9] Conventionally, in hospital settings, about 10 mL of blood from the patients is cultured in blood culture incubators using specific media to culture either aerobic or anaerobic organism (Figure 1).[5] If the blood culture is positive, then pathogen identification can be determined by follow-up

pathogen cultures including Sabouraud glucose agar and cornmeal agar.[5] Chromogenic medium-based culture can further improve *Candida* differentiation as the medium contains chromogenic substrates that react with enzymes produced by different pathogens and produce colonies of varying colors and morphologies.[10] Additionally, carbohydrate assimilation and fermentation reactions can be used for *Candida* identification, however, the test takes up to 72 h incubation. Overall, the conventional laboratory methods for *Candida* detection are highly time-consuming, which delays patient treatment and can take 2−10 days.

It is important to rapidly diagnose fungal infections with high accuracy to initiate timely treatment. The nucleic acid real-time polymerase chain reaction (PCR), nucleic acid sequence-based amplification (NASBA), and loop-mediated isothermal amplification (LAMP) methods take around 3−6 h for detection of *C.albicans*. Microfluidic devices have emerged as a potential candidate for rapid disease diagnostics in the current era.[11−19] Although, microfluidic real-time PCR
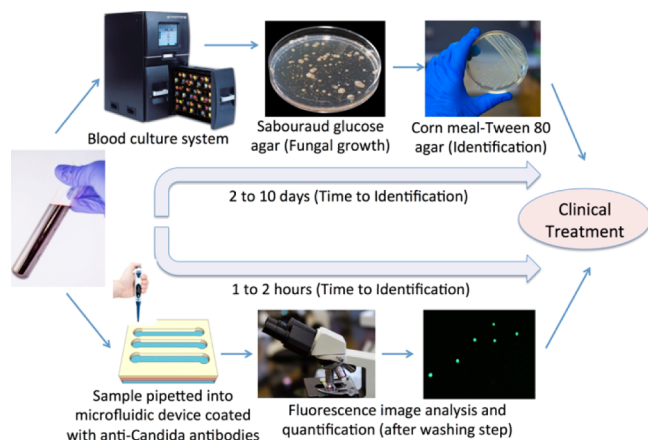
Figure 1. Comparison of the conventional culture method and the microchip-based *C. albicans* detection. In conventional procedure for *Candida* detection in clinical settings, the following protocol is followed; (1) blood sample is collected from patients. (2) Blood samples are incubated in an automated blood culture system. (3) Pathogens are grown on a Sabouraud glucose agar plate. (4) Sample is subcultured into a Corn meal-Tween 80 agar plate for morphological identification. In the point-of-care testing approach, the following protocol is developed; (1) blood sample collection [spiked with green fluorescent protein (GFP)-expressing *Candida* as a model microorganism]. (2) Blood sample is analyzed in microchannels functionalized with anti-*Candida* antibodies. *Candida* cells were specifically captured by antibodies on the microchannel surface. Unbound *Candida* cells are washed away with PBS in the washing step. (3) GFP-*Candida* is imaged and quantified under a fluorescence microscope. Some images are taken from freerangestock.com and pixabay.com.

provides a rapid *Candida* detection,[20] it relies on thermal cycles and the effective monitoring and control of various temperatures during experimentation are quite challenging. The fluidic manipulations and utilization of magnetic beads also increase the complexity of the devices. Table 1 shows comparison of different *Candida* detection technologies.

To overcome the limitations of conventional culture-based *Candida* detection methods, we developed an inexpensive microfluidic device functionalized with antibodies to *C. albicans*. This technology allows for rapid detection, capture, and isolation of *C. albicans* in PBS and blood in an efficient manner. Low sample consumption because of the higher surface to volume ratio of blood within the microchannels supports rapid capturing of targeted pathogens. Our device overcomes the limitation of long incubation times. We demonstrated that *C. albicans* can be detected within 2 h at a minimum of 10 cfu/mL.

2. MATERIALS AND METHODS

2.1. Microfluidic Device Fabrication. The microfluidic device was fabricated by using plastics layers and polymer adhesives as previously reported (Figure 2a,b).[17] The design for the device was created in AutoCAD 2015 and uploaded to the UCP Software for cutting the device using a laser cutter. Poly(methyl methacrylate) (PMMA) (McMaster-Carr, Atlanta, GA and ePlastics, San Diego, CA 1.5 mm thick) and the double-sided adhesive (DSA) (3 M, St. Paul, MN, 76 µm thick) were cut using a VLS 2.30 laser cutter (VersaLaser, Scottsdale, AZ). In each microfluidic device, three parallel channels (dimensions: 44 mm × 5 mm × 76 µm) were cut in DSA. One side of the DSA film was attached to glass cover

Table 1. Comparison of Developed Technology with Existing Methods of *Candida* Detection

| method	sensitivity	assay time	limit of detection	description
blood culture-based detection	42–60%[21,22]	1–7 days	1 cfu/mL[23]	it is time consuming (takes several days) and labor-intensive method that may delay treatment options. Blood culture-based methods require large volumes of blood
PCR	85–90%[24]	4–6 h	≤10 cfu/mL[23]	PCR is very sensitive method and provides low limit of detection. This method requires breaking of fungal cell wall. The difficulty in breaking *Candida* cell wall makes the DNA extraction step quite difficult. Furthermore, PCR is highly temperature dependent process, thus requires strict temperature control and thermal cycling, which makes PCR unsuitable for POC settings
NASBA	higher than 90%	~2–3 h	1–10 cfu/mL[25,26]	it is an isothermal amplification process that eliminates the need of thermocycler.[25] It reduces the *Candida* detection time as compared to PCR and other culture-based methods. NASBA has quicker nucleic acid extraction as compared to PCR based methods.[27] As this method requires nucleic acid extraction and purification, hence has limited application in POC settings
LAMP	similar to NASBA and PCR method[28]	~2–3 h	similar to NASBA and PCR[29]	LAMP provides a cost-effective and rapid isothermal amplification method for the detection of *C. albicans*.[30] Similarly to NASBA, it requires nucleic acid extraction and purification step, hence has limited application in POC settings
T2Candida	91.6%[31]	3–5 h	1–3 cfu/mL	it can rapidly detect the *C. albicans* from blood samples. The detection time is 3–5 h. However the system is costly
Developed method	not tested with clinical samples	~2 h	$10–10^5$ cfu/mL	the developed assay can be used as a cost-effective and rapid *Candida* detection method at POC as well as clinical settings. It requires only 50 µL sample volume and up to 2 h maximum for detection of *Candida*. It can also work with larger volumes with extended assay times.
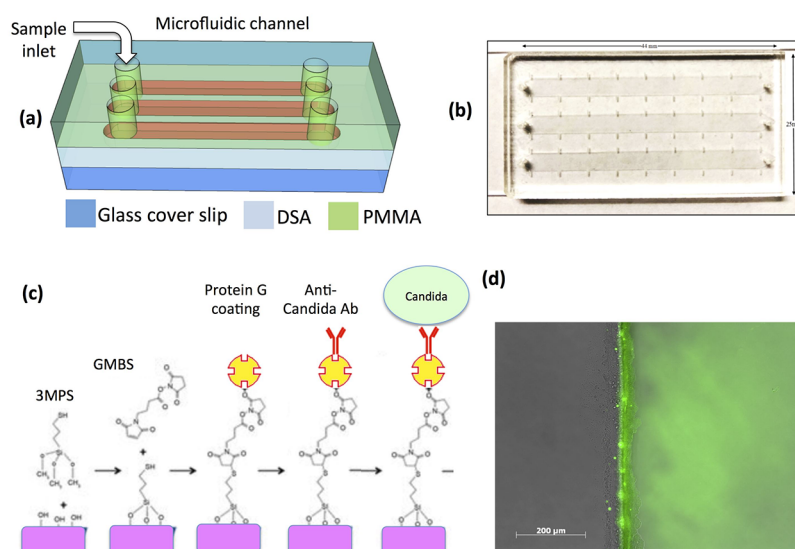
Figure 2. (a) Schematic of the microfluidic chip consisting of PMMA, DSA, and glass cover. (b) Actual image of the assembled microchip containing three microfluidic channels, inlets, and outlets. (c) Protein G-based surface chemistry was used to immobilize anti-*Candida* antibodies on the surface. (d) Fluorescent image showing clear green signal (right side of microchannel) that indicates successful immobilization of fluorescein isothiocyanate (FITC)-conjugated protein.

slide [70% ethanol in distilled (DI) water and dried by nitrogen gas], whereas other side was attached to a PMMA. Three inlet and three outlet holes (0.65 mm diameter) were also cut in PMMA before assembly. The complete assembled device is shown in (Figure 2b).

2.2. Microfluidic Channel Surface Functionalization with Antibodies. Protein G-based surface chemistry was used to immobilize antibodies (Figure 2c). Protein G is an immunoglobulin-binding protein that binds to the fragment crystallization region of antibodies with high efficiency. For surface functionalization, glass cover slide was cleaned with 70% ethanol in DI water and dried by nitrogen gas. Glass cover slide was then treated with oxygen plasma (100 W, 1% oxygen) for 2 min in a PX-250 chamber (March instruments, Concord, MA) to form the hydroxyl (OH) surface functional groups followed by a 30 min incubation with silanization solution [4% (v/v) 3-MPS ((3-mercaptopropyl)trimethoxysilane, CN: 175617)) in ethanol] in a Petri dish at room temperature for covalent binding. After incubation, the cover slide was washed with ethanol and was allowed to dry for 3−4 min at room temperature. The microfluidic device was assembled by sandwiching DSA between PMMA and cover slide. Channels were washed 3 times with PBS. GMBS (N-g-maleimidobutyr-yloxy succinimide ester) solution (4% (w/v) GMBS dissolved in 10% DMSO in PBS) was pipetted into microfluidic channels. Devices were incubated for 30 min at room temperature. From now onward, channels were washed 3 times with PBS after each incubation step. Then Protein G (1 mg/mL in PBS, Thermo Fisher Scientific) was pipetted into microfluidic channels followed by 2 h incubation at 4 °C. For capturing *C. albicans*, we tested two different anti-*Candida* antibodies; one was monoclonal (Abcam, ab82704) and the second was polyclonal (Thermo Fisher Scientific, Catalog: PA1-27158). Then 30 μL (5 μg/mL solution) of each anti-*C. albicans* antibody was pipetted into each microchannel followed by a 1 h incubation at room temperature. Microchannels were washed with PBS 3 times. Then 2% (w/v) bovine serum albumin in PBS was injected into microchannels followed by 30 min incubation at room temperature and

subsequent washing with PBS was performed. The devices were ready for *Candida* capture experiments.

2.3. *C. albicans* Strain and Growth. To validate the surface chemistry and isolation experiments, a genetically modified *C. albicans*, SC5314, expressing GFP was used.[32] *C. albicans* was grown to the log phase in yeast extract−peptone−dextrose medium overnight at 30 °C in a shaker incubator at 250 rpm.[33] Yeast was harvested, washed, and resuspended in PBS and blood for use. The initial *Candida* count was determined first by haemocytometer. Then, *C. albicans* were thoroughly mixed with PBS/blood to obtain a homogeneous solution. After *Candida* capture experiments, counting was performed manually. The counting approach was optimized (Figure S1). Capture efficiency was calculated by dividing the number of *Candida* cells captured by number of *Candida* cell spiked. Only 50 μL of the *Candida*-spiked sample was used for these experiments. *Candida* counts were normalized to the sample volume used.

2.4. Sample Preparation for Microfluidic Experiments. GFP-expressing *C. albicans* (GFP-*Candida*) was spiked into 1× PBS and whole blood with the final concentrations ranging from 10 to 5×10^5 cfu/mL for analysis on the chip. Discarded deidentified whole blood (purchased from Research Blood Components, LLC, Cambridge, MA) from healthy individuals was used in this study. For lysed blood experiments, we did the following: (1) GPF-*Candida* cells were spiked into whole blood at 5×10^5 cfu/mL and mixed thoroughly by inverting to enable homogenous distribution. (2) The spiked blood sample was mixed with ACK (ammonium−chloride−potassium) lysis buffer at 1:10 ratio (v/v) (Thermo Fisher Scientific, A1049201) and incubated for 3 min at room temperature. *Candida* cells remained intact in ACK lysis buffer. This is primarily because of the chitin found in their cell wall.[34] (3) Centrifugation at 3000 rpm for 3 min was performed; *Candida* cell remained intact and made a pellet. (4) Supernatant was aspirated, leaving approximately 50 μL to avoid disturbing the pellet. Then, 5 mL of PBS was added followed by 3000 rpm centrifugation (3 min) and the supernatant was aspirated again leaving approximately 50 μL
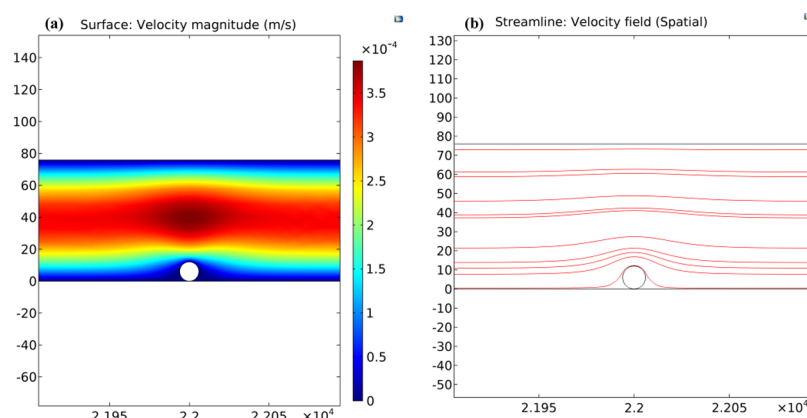
Figure 3. COMSOL simulation results for 5 μL/min. (a) Simulated flow velocity representation inside the microfluidic device. (b) Streamlines for the velocity field.
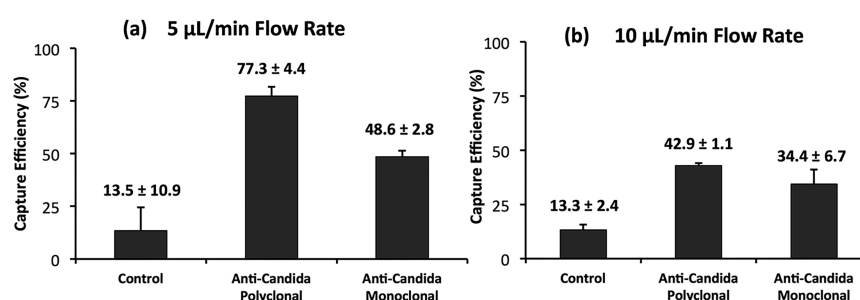

Figure 4. Comparison of the capture efficiency of *C. albicans* by two different anti-*Candida* antibodies; polyclonal and monoclonal. The *Candida* sample was incubated inside microchannels to allow binding with a functionalized surface for 15 min at room temperature. (a) Capture efficiency was significantly higher when the washing step was performed at (a) 5 μL/min as compared to (b) 10 μL/min.

of the sample including pellet. (5) The pellet was disturbed with a pipette and mixed gently. The complete blood lysis and *Candida* enrichment process take about 10 min.

2.5. Operation of Microfluidic Experiments, *Candida* Capture, and Quantification. To optimize the capture efficiency, 50 μL of the GFP-*Candida* sample was pipetted into the functionalized microchannels, and then incubated at ambient temperature for 15 min. Following the incubation, microchannels were washed with PBS at a flow rate of 5 or 10 μL/min using a syringe pump (Harvard Apparatus, Holliston, MA) for 60 min. After washing, captured GFP-*Candida* was imaged using an inverted fluorescence microscope (Zeiss Observer optical microscope) through a GFP fluorescence filter (excitation wavelength 470 nm). For comparison, bright-field images were also taken (Figure 5c). All images were taken (Figure 5c and 5d) with 10× objective except Figure 5e (100× objective). The number of GFP-*Candida* detected using a GFP filter was counted manually.

2.6. Statistical Analysis. Statistical analysis was performed using one-way analysis of variance (ANOVA). Each experiment was repeated at least three times. A *p*-value of less than 0.05 was considered statistically significant.

3. RESULTS

We developed a microfluidic device having 3 microchannels functionalized with protein G-based surface chemistry (Figure 2). The microfluidic channels provide a high surface to volume ratio that would allow efficient *Candida* capture on the surface of the channel. 3MPS−GMBS (3-mercaptopropyl trimethoxysilane-*N*-*g*-maleimidobutyryloxy succinimide ester)-based surface chemistry was used to immobilize protein on the

microchannels of the device as previously reported.[17] The reaction between the amine group and GMBS allow protein immobilization. To determine protein conjugation to the surface of microchannels, we incubated FITC-conjugated protein inside microchannel after the GMBS step. The channels were washed with PBS after 2 h of incubation at 4 °C. We visualized fluorescence using the fluorescent microscope, which showed that protein was successfully immobilized inside microchannels (Figure 2d).

COMSOL simulations were performed to determine the effects of shear stress on the *C. albicans* captured inside the microfluidic device. A single *Candida* cell was modeled as a spherical-shaped object (radius 6 μm). The microfluidic device with exact dimensions was considered and a laminar flow was assumed. The no slip boundary condition was applied to the walls of the microfluidic channel. Two flow rates (5 and 10 μL/min) were assumed. A boundary condition with pressure = 0 was set for the outlet. The Naiver−Stokes equation was used to simulate the motion of fluid past the captured *Candida*. Various simulations were carried out to calculate the velocity and pressure profiles. The velocity magnitude and streamlines are shown in Figure 3a,b, respectively. The shear stress was measured for the two flow rates (5 and 10 μL/min) and drag force was also calculated. The value of drag force was 20.47 pN for 5 μL/min. When the flow rate was increased to 10 μL/min, drag force became 40.96 pN. Higher flow rates resulted in increased drag force on the captured *Candida*. This increase in drag force reduces the capture efficiency of *Candida* as also observed in experiments (Figure 4).

We evaluated two different antibodies (monoclonal and polyclonal) for their efficiency to capture *C. albicans* from
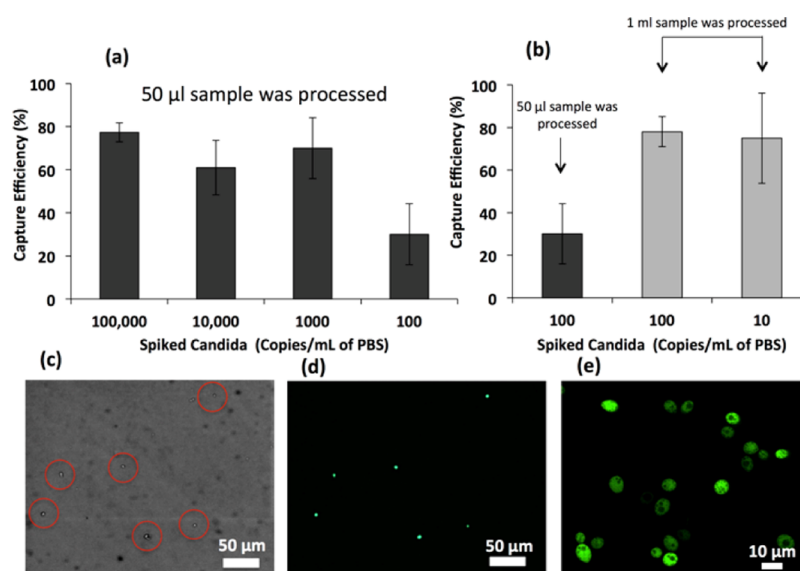
Figure 5. (a) Capture efficiency of *C. albicans* cells inside microfluidic channels functionalized with polyclonal antibodies at various concentrations ranging from 100 to 100 000 cfu/mL. We processed 50 μL of spiked PBS. (b) Capture efficiency of *Candida* was increased when 1 mL of the spiked PBS sample was processed by injecting 50 μL sample multiple times in the same channel followed by incubation after each injection. (c) Image of the captured GFP-*Candida* inside microchannel at 10× magnification under bright field. (d) Image of the captured GFP-*Candida* inside microchannel at 10× magnification under a fluorescence microscope. (e) Image of the captured GFP-*Candida* at 100× magnification under a fluorescence microscope.

spiked samples inside microfluidic channels. The capture efficiency via polyclonal anti-*Candida* antibodies (77.4 ± 4.4%) was observed to be significantly higher (*p*-value < 0.05) than monoclonal anti-*Candida* antibodies (48.6 ± 2.8%) (Figure 4a). For all further experiments, we used polyclonal antibody because of its higher capture efficiency compared to monoclonal.

We observed that the 5 μL/min flow rate (during washing) gave significantly higher capture efficiencies compared to 10 μL/min (Figure 4). The lower efficiency observed at higher flow rates may be related to the correspondingly higher shear stress within the microchannels as also shown in simulation graphs (Figure 3). For all further experiments, we used a flow rate of 5 μL/min.

To determine microchip's limit of detection for *Candida* capture, we spiked GFP-*Candida* into PBS at various clinically relevant concentrations ranging from 10 to 10^5 cfu/mL (Figure 5).[35−37]

In the first set of experiments, we tested only 50 μL of the spiked PBS sample. We observed similar capture efficiencies of 77.4 ± 4.4, 61 ± 12.7, and 70 ± 13.2% for 10^5, 10^4, and 10^3 cfu/mL samples respectively, however, capture efficiency was reduced to 30 ± 14.2% for the 10^2 cfu/mL sample. One possible reason for lower capture efficiency in the case of the 10^2 cfu/mL sample may have originated from losing *Candida* during sampling from 1 mL to 50 μL. To investigate this hypothesis, we tested whole 1 mL of spiked samples and observed higher capture efficiencies of 78 ± 13.2 and 75 ± 21.1% for 10^2 and 10 cfu/mL samples (Figure 5b). From these results, we observed that increasing the sample volume resulted in increase in capture efficiencies at lower concentration samples (10 and 10^2 cfu/mL).

To further investigate the effect of the sample matrix and the presence of other cells on the capture efficiency, we spiked 10^5 cfu/mL GFP-*Candida* into whole human blood and processed the sample using the microfluidic device. We observed the

capture efficiency of 40.5 ± 4.7% from blood samples (Figure 6), which was significantly lower than when spiked PBS was used 77.4 ± 4.4%. This decrease in capture efficiency from the blood sample may be due to the presence of millions of blood cells that hindered *Candida*−antibody interactions. To overcome the effect of blood cells, we lysed the spiked blood sample and isolated the pellet as described in the Materials and Methods section. The pellet containing *Candida* cells was
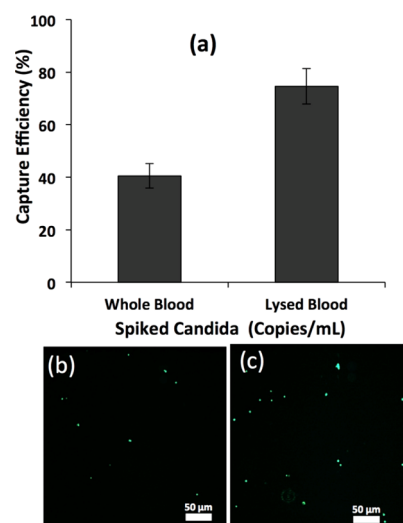

Figure 6. (a) Capture efficiency of GFP-*C. albicans* cells inside microfluidic channels functionalized with polyclonal antibodies from 16 μL of whole and lysed blood. Blood was lysed after spiking GFP-*Candida*. (b) Image of the captured GFP-*Candida* from whole blood inside the microchannel at 10× magnification under a fluorescence microscope. (c) Image of the captured GFP-*Candida* from lysed blood inside microchannel at 10× magnification under a fluorescence microscope. GFP-*Candida* was spiked into blood at the concentration of 10^5 cfu/mL.

mixed and processed through the microfluidic device. We observed significantly higher capture efficiency of 74.6 ± 6.8% compared to the spiked whole blood sample.

To show that the developed microfluidic device can be used to capture unstained *Candida* from samples, unstained *Candida* spiked in buffer (not expressing GFP) was utilized. Precapture and postcapture staining were performed with FITC conjugated anti-*Candida* antibody (ab21164). The results are shown in the Figure S2. *C. albicans* not producing GFP were initially captured using the polyclonal antibody. Then staining was performed with FITC-conjugated anti-*Candida* antibody. The capture efficiency was recorded 68.8 ± 6.8%. It was observed that precapture staining resulted in lower capture efficiency, that is, 43.2 ± 4.5%. One possible reason can be the blocking of some capture sites of the *Candida* strains. The *C. albicans* captured by monoclonal antibody in the microfluidic channel were also stained with the secondary antibody. The postcapture staining resulted in a capture efficiency of 29.6 ± 5.7%. The precapture staining resulted in slightly decreased capture efficiency 22.4 ± 4.5%.

4. DISCUSSION

Using the developed microfluidic device, we were able to efficiently isolate and quantify *C. albicans* from spiked PBS and whole blood sample. The whole capture experiment takes about 1.5 h (in the case of PBS and whole blood) and less than 2 h (in the case of blood lysis protocol). Following blood lysis protocol developed herein, we were able to capture 74.6 ± 6.8% of *Candida* from blood samples in significantly lesser time (2 h) as compared to conventional blood culture followed by plate culture methods (more than a week) (Figure 1). There are other existing methods that can detect *Candida* at lesser time than conventional culture methods including PCR,[38] magnetic resonance (T2Candida by T2 Biosystems),[3,39] and isothermal amplification methods such as LAMP,[40,41] NASBA,[42] and rolling circle amplification.[43] PCR and isothermal amplification-based detection provide high specificity and sensitivity; however, these approaches are multistep, require sample purification, and nucleic acid extraction prior to the detection step, a time-consuming process. More importantly, during nucleic acid amplification *Candida* cells are lysed, hence viability of *Candida* cannot be tested and drug resistance and susceptibility cannot be analyzed. The T2Candida system utilizes magnetic resonance to detect aggregation of magnetic particles in the presence of the target.[39] T2Candida also relies on amplification of genetic information where nucleic acids are first isolated and purified from *Candida* cells using beads; however, T2Candida is a sample-to-answer system where all the processing steps are automated. This system provides high sensitivity and specificity and detection can be performed in 3–5 h.[3] However, similar to other nucleic acid-based detection systems, *Candida* cells are lysed in T2Candida and drug resistance and susceptibility testing cannot be performed, which are becoming very important for personalized therapy. The developed microfluidic device can address some of these limitations by allowing whole *Candida* capture directly from the lysed blood sample as *Candida* cells are not lysed during the isolation step, hence culture and drug resistance and susceptibility testing may be possible.

Although blood is a complex biological matrix; we have successfully captured and detected spiked *Candida* using the microfluidic approach. The presented method can also be applied to other matrices such as saliva and urine. In the case of urinary tract infection, high concentration of *Candida* cells (10^4 to 10^5 cfu/mL)[37] may be present in urine that can be rapidly isolated and quantified using the developed microfluidic devices. In this study, GFP-expressing *C. albicans* was used to facilitate the detection and quantification under the fluorescent microscope. The extension from a GFP-expressing *Candida* strain to wild-type strains can be simply achieved using an ELISA or peptide nucleic acid-based fluorescence in situ hybridization (PNA-FISH)-based detection method, as previously reported.[44−46] The characterized microchip can be integrated with rapid detection methods such as lensless imaging[17,47] and smartphone-based imaging[48−50] to enable point-of-care testing. The presented microfluidic approach can be adapted to detect other microorganisms that cause sepsis such as Gram-negative and Gram-positive bacteria.[51] Also, the cost to fabricate one functionalized chip is significantly lower than other assays (PCR, T2Candida); current material cost (excluding other related costs such as labor and equipment cost) includes 10¢ of glass, 1¢ of PMMA, and 80¢ of antibodies per device. The antibody cost could be lowered with large-scale production and ordering.

5. CONCLUSIONS

To overcome the limitations of culture-based detection methods for fungal infections, we have developed a microfluidic immunoassay to capture *C. albicans* (a Yeast) from PBS and blood samples with reliable capture efficiency. We observed that polyclonal antibody captured a significantly high number of *Candida* cells as compared to monoclonal antibody. The washing flow rate can also affect final capture efficiency, and we observed that a flow rate of 5 μL/min provides higher capture efficiency as compared to 10 μL/min. To enable efficient detection of *Candida* from blood samples, the lysis step was used that significantly improved the capture efficiency from whole blood samples. The presented technology allows the capture and isolation of whole *Candida* cells, hence enabling potentially drug resistance and susceptibility testing. The microfluidic platform can be potentially adapted to detect various other microorganisms and pathogens rapidly at the point-of-care settings.

■ ASSOCIATED CONTENT

ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsomega.9b00499.

> *Candida* quantification process and *Candida* capture efficiency (PDF)

■ AUTHOR INFORMATION

Corresponding Authors
*E-mail: wasghar@fau.edu (W.A.).
*E-mail: utkan@stanford.edu (U.D.).
ORCID ⓘ
Waseem Asghar: 0000-0003-0458-8430
Utkan Demirci: 0000-0003-2784-1590
Notes
The authors declare the following competing financial interest(s): U.D. is a founder of, and has an equity interest in (i) DxNow Inc., a company that is developing microfluidic

and imaging technologies, (ii) Koek Biotech, a company that is developing microfluidic IVF technologies for clinical solutions, and (iii) LEVITAS Inc., a company that develops biotechnology tools for genomic analysis in cancer. U.D.'s interests were viewed and managed in accordance with the conflict of interest policies. All other authors declare no conflict of interest.

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